ORIGINAL ARTICLE

Evaluation of clonal relatedness of extended-spectrum β-lactamase-producing *Proteus mirabilis* isolates by quantitative antibiogram and RAPD typing

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Objective: To delineate, using two different typing systems, the clonal relatedness of 40 isolates of extended-spectrum β-lactamase (ESβla)-producing *Proteus mirabilis* obtained over a period of 7 years in six hospitals in the Paris area and two in Pas-de-Calais.

Methods: Random amplified polymorphic DNA (RAPD) polymerase chain reaction typing was applied by using three random primers on the ESβla-producing *P. mirabilis* isolates and on isogenic *Escherichia coli* strains with or without plasmids encoding the representative resistance pattern transferred from *P. mirabilis*. Quantitative antibiogram typing, which was also applied to the *P. mirabilis* isolates, was used to define the euclidean distance between these strains.

Results: After having demonstrated that *P. mirabilis* plasmids did not influence chromosomal DNA amplification, we could classify the ESβla-producing *P. mirabilis* isolates into 12 groups based on RAPD fingerprints. The same isolates were classified into 19 groups by quantitative antibiogram typing. Despite this difference in group numbers, general concordance between the typing systems was observed. This allowed us to show that the greater number of isolates in some hospitals belonged to a single strain and that single isolates obtained in different hospitals generally represented unique strains.

Conclusions: A small number of ESβla-producing *P. mirabilis* strains was isolated during 7 years in the eight medical centers studied, and the number of different strains identified suggested that inter-hospital transfer had not occurred.

Key words: Extended-spectrum β-lactamase-producing *P. mirabilis*, quantitative antibiogram typing system, arbitrarily primed PCR typing system

INTRODUCTION

Resistance to third-generation cephalosporins by production of extended-spectrum β-lactamases (ESβla) emerged in France in the mid-1980s in different enterobacterial species [1–5] but only at the end of that decade in *Proteus mirabilis* [6,7]. Moreover, the clinical occurrence of such *P. mirabilis* isolates has remained rare since the appearance of the first strain in 1987 in France. Therefore, we collected all the ESβla-producing *P. mirabilis* isolates from 1987 to 1993 in different hospitals in the Paris area and in a hospital in Pas-de-Calais. Further strains which were isolated in a rehabilitation institute in Pas-de-Calais within a 3-month period in 1992 were also included in the study. Since the transfer of patients between hospitals in the same area occurs frequently and could be a cause of the spread of ESβla-producing *P. mirabilis*, as was described for ESβla-producing *Klebsiella pneumoniae* [8–10], the goal of this study was to type all these isolates in order to delineate their clonal relatedness.

Two typing systems were used, the phenotypic quantitative antibiogram method described by Blanc et al. [11] and random amplified polymorphic DNA

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(RAPD) fingerprinting [12]. The first method was chosen because it can be considered as a universal method, since any microbiological laboratory can apply it. The second one was chosen because it represents the most rapid method among the DNA-based typing systems and seems, according to the study of Bingen et al. [13], a promising method for *P. mirabilis* typing. Nevertheless, before applying RAPD fingerprinting to ESβla-producing *P. mirabilis*, we applied it to the *Escherichia coli* derivatives expressing the transferable antibiotic resistance patterns from the *P. mirabilis* strains in order to confirm, as previously described [14,15], that the antibiotic resistance-encoding plasmids do not change the RAPD fingerprints of the isogenic plasmid-free *E. coli*.

MATERIALS AND METHODS

Bacterial strains

All consecutive but not repetitive ESβla-producing *P. mirabilis* isolates obtained between 1987 and 1993 in six Parisian hospitals (A, B, C, D, E and F) and a hospital in Pas-de-Calais (G) were included. The five isolates (s1–5) which were previously demonstrated as being responsible for an outbreak in hospital B in 1989 [7] were used in this study as a reference set of epidemiologically related strains. Thirteen further strains were provided by a rehabilitation institute (H) in Pas-de-Calais, in which an outbreak of infection by ESβla-producing *P. mirabilis* had been ongoing since November 1992. For the hospitals where several strains were collected (A, n=13; B, n=8; C, n=2), the isolation dates and the wards in which the patients were situated are reported in Table 1. In each of the remaining hospitals, D, E, F and G, only one strain was isolated in 1987, 1992, 1993 and 1989, respectively.

E. coli J53-2 (F–, *met* F63, *pro* B22, *rif*^{R}) was used as recipient in conjugation experiments, while *E. coli* MC-1061 [*recA*, *hsdR mcrB araD* 139 Δ(*ara ABCleu*)7679 Δ*Xlac*74 *galU galK rpsL thi*)] served for transformation by electroporation.

Susceptibility testing

Susceptibility to 22 antibiotics (amoxicillin, co-amoxiclav, ticarcillin, cephalothin, cefamandole, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, kanamycin, gentamicin, tobramycin, netilmicin, amikacin, chloramphenicol, tetracycline, fosfomycin, sulfamethoxazole, trimethoprim, co-trimoxazole and pefloxacin) was determined by the disk diffusion agar method on Mueller-Hinton agar (bioMérieux, Marcy l'Etoile, France) and interpreted in accordance with the recommendations of the Antibiogram Committee of the French Society for Microbiology [16]. As previously described, the production of ESβla was detected by the double disk synergy test [17].

Plasmid transfer

Plasmid-mediated antibiotic resistance was transferred by conjugation experiments as previously described [7] or by electroporation according to standard procedure [18]. *E. coli* derivatives were selected on agar containing 150 mg/L ampicillin (Bristol-Myers-Squibb, Paris, France), 0.5 mg/L cefotaxime (Hoechst-Marion-Roussel, Paris, France) or 25 mg/L kanamycin (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France).

Table 1 Origin of 23 ESβla-producing isolates of *P. mirabilis* obtained in three hospitals in the Paris area (1987–1993)

Hospital A			Hospital B			Hospital C		
Isolate number	Isolation		Isolate number	Isolation		Isolate number	Isolation	
	Ward	Date[a]		Ward	Date		Ward	Date
1	icu	05.90	13	icu	09.89	16	ortho	12.91
2	icu	12.90	s1	icu	11.89	30	ortho	03.93
3	icu	03.91	s2	icu	11.89			
4	icu	03.91	s3	icu	11.89			
5	icu	05.91	s4	icu	12.89			
6	icu	06.91	s5	icu	12.89			
7	reh 1	06.91	15	ortho	08.91			
8	icu	09.91	12	icu	08.91			
29	ped	03.93						
91	reh 2	06.93						
92	reh 2	07.93						
93	reh 2	08.93						
95	reh 2	09.93						

Abbreviation: icu=intensive care unit, ped=pediatrics, ortho=orthopedics, reh=rehabilitation unit.
[a]Month and year of strain isolation.

Rifampin (Hoechst-Marion-Roussel, Paris, France) at a concentration of 150 mg/L was added to the selecting medium in the conjugation experiments.

Quantitative antibiogram typing

Similarity analysis of the 40 ESβla-producing *P. mirabilis* strains was performed on the basis of the antibiotic susceptibility by using the euclidean distance. The antibiotics used in this analysis were those which showed the greatest variability of inhibition zone diameters and for which the resistance mechanisms were encoded by plasmid or chromosomal genes: cefotaxime, ceftazidime, gentamicin, amikacin, chloramphenicol, trimethoprim and pefloxacin. The cut-off distance below which discrepancies were due to causal variability was calculated by measuring the inhibition zone diameters for each of the 40 strains on two different occasions. The cut-off distance was defined so that >95% of the distances between the first and the second determinations would be smaller than the cut-off distance. Thus, two isolates were considered to be different when the euclidean distance between them was higher than the cut-off value. The algorithm used to produce a dendogram for antibiogram typing was the unweighted per group method [19].

Random amplified polymorphic DNA (RAPD) fingerprinting

Bacteria were grown for 18 h on Columbia agar medium supplemented with 5% sheep blood. A colony was suspended in 0.5 mL of lysis buffer (20 mM Tris-HCl (pH 8.3) at 20°C, 50 mM KCl, 0.1% Tween-20), heated at 96°C for 10 min, and then maintained at room temperature until used in the reaction mixture.

Three primers were used: AP4 (5′-TCACGATGCA-3′) [12], HLWL74 (5′-ACGTATCTGC-3′) [20] and MHN1 (5′-ACGTCTATGC-3′). The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 4 μmol/L of primer, 400 μmol/L of each deoxynucleoside triphosphate (Pharmacia), 0.5 U of *Taq* DNA polymerase (Perkin-Elmer-Cetus), and 1 μL of extract in lysis buffer in a final volume of 25 μL. Samples were subjected to 45 cycles in a GeneAmp PCR9600 thermal cycler (Perkin-Elmer) under the following conditions: denaturation, 94°C for 15 s; annealing, 36°C for 15 s, extension, 72°C for 70 s. Final extension was 5 min at 72°C. Polymerase chain reaction (PCR) products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Two fingerprints were defined as different when they differed from each other by at least a high-intensity band, or a low-intensity band which was reproducible in three independent amplification experiments.

RESULTS

Antibiotic susceptibility of ESβla-producing *P. mirabilis* and their *E. coli* derivatives

The 40 collected *P. mirabilis* isolates exhibited β-lactam susceptibility profiles consistent with the production of

Table 2 Resistance patterns of *E. coli* derived from ESβla-producing *P. mirabilis*

		E. coli derivative	
Hospital	*P. mirabilis* isolate number	Resistance markers co-transferred by conjugation	Resistance pattern
A	1, 2, 3, 4, 5, 6, 8, 29	ESβla/kgtn/cmp/sulf	I
	7	ESβla/kgtn/cmp/sulf/tmp/sxt	II
	91	No transfer	
	92, 93, 95	ESβla/ktna/sulf	III
B	s1, s2, s3, s4, s5, 15	ESβla/ktna/sulf	III
	12	No transfer	
	13	ESβla/kgt/sulf/tmp/sxt	IV
C	16	ESβla/kgtn/cmp/sulf	I
	30	ESβla/ktna/cmp/sulf/tmp/sxt	V
D	14	ESβla/ktna/sulf/tet[a]	VI
E	17	ktna/cmp/sulf	VII
F	90	ESβla/kgtn/sulf	VIII
G	89	No transfer	
H	18, 20, 31, 32, 33, 34, 36, 44	ESβla/kgtn/sulf	VIII
	19, 21, 22, 35, 45	ESβla/gtn/sulf/tmp/sxt	IX

Abbreviation: ESβla=extended-spectrum β-lactamase: a=amikacin, cmp=chloramphenicol; g=gentamicin; k=kanamycin; n=netilmicin; sulf=sulfamethoxazole; sxt=co-trimoxazole; t=tobramycin; tet=tetracycline; tmp=trimethoprim.

[a] *E. coli* derivative obtained by electroporation.

ESβla: (1) decreased susceptibility or resistance to amoxicillin, ticarcillin, cephalothin, third-generation cephalosporins and aztreonam; (2) full susceptibility to cefoxitin and imipenem, and (3) a positive disk synergy test with a co-amoxiclav disk and third-generation cephalosporin disks. Except for strains 12 and 13, the inhibition zone diameters of cefotaxime were smaller than those of ceftazidime and aztreonam (data not shown). Different multiple aminoglycoside resistance patterns were observed and each of them was transferable into *E. coli* when the mating experiments were successful (Table 2). All the *P. mirabilis* isolates were resistant to sulfamethoxazole but were distinguished by different susceptibilities to chloramphenicol, trimethoprim, co-trimoxazole, fosfomycin and pefloxacin (data not shown). The transferability of these differential antibiotic markers into *E. coli* is indicated in Table 2. The antibiotic resistance transfer failed from three ESβla-producing *P. mirabilis* isolates (12, 89 and 91) whichever transformation method or antibiotic selector (ampicillin, cefotaxime or kanamycin) was used. The transfer of the ESβla produced by *P. mirabilis* strain 17 was not successful, whereas that of other plasmid-mediated resistance markers was (Table 2). Finally, nine transferable antibiotic resistance patterns were obtained in the 37 *E. coli* derivatives.

The transfer from 12 of the 13 ESβla-producing *P. mirabilis* strains isolated in hospital A resulted in three patterns (I, II, III) in the *E. coli* derivatives. Two of these three patterns were also observed in *E. coli* derived from *P. mirabilis* isolated in other hospitals, namely pattern III in *E. coli* strains derived from six of the eight ESßla-producing *P. mirabilis* strains isolated in hospital B and pattern I in the *E. coli* strain derived from one of the two *P. mirabilis* strains isolated in hospital C. From the 13 *P. mirabilis* strains isolated in hospital H, two new different transferable resistance patterns, VIII and IX, were observed, but pattern VIII was also observed in the *E. coli* strain derived from the single *P. mirabilis* strain isolated in hospital F. The four remaining patterns (IV, V, VI and VII) were each observed in *E. coli* strains derived from a single ESβla-producing *P. mirabilis* isolate.

Quantitative antibiogram typing

The determinations on two occasions of the antibiotic susceptibility of each ESβla-producing *P. mirabilis* isolate showed that the greatest euclidean distance was 11.4, and 95.4% of the same isolates retested had a difference of less than 11. Therefore, a cut-off at a distance of 11 was defined, above which discrepancies were considered as reflecting differences between the isolates. The dendogram based on the quantitative antibiogram typing classified the 40 ESβla-producing *P. mirabilis* isolates into 19 groups (Figure 1).

The 13 strains isolated in hospital A between 1990 and 1993 were classified into seven groups. According to the cut-off distance, seven of these strains (1, 2, 3, 4, 5, 6 and 8) were found to be identical and constituted the first group. All these strains were isolated in the

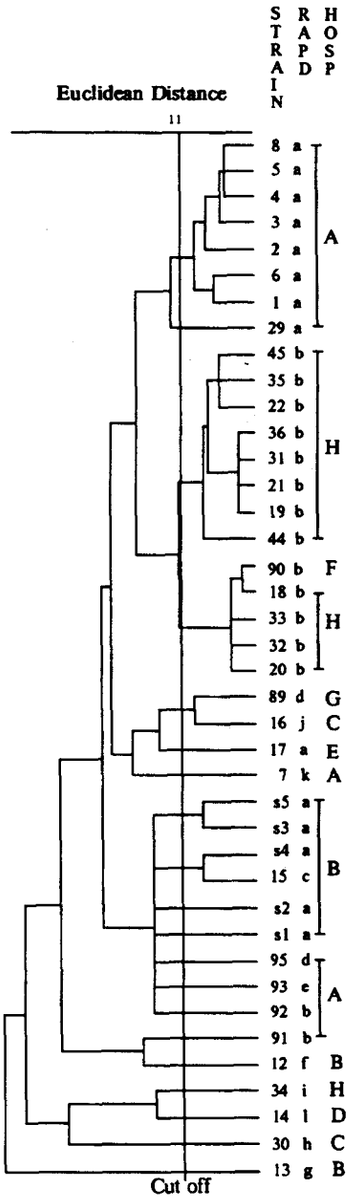

Figure 1 Dendogram of 40 ESβla-producing *Proteus mirabilis* isolates obtained by quantitative antibiogram typing and their RAPD fingerprints. The hospitals where these strains were isolated are indicated in the right-hand lane.

same intensive care unit (ICU) but during a period of 16 months (Table 1). The second group, which included only one strain (29), was closely related to the first group. Each of the five remaining strains isolated in hospital A was placed in a specific group (Figure 1).

The eight strains isolated in hospital B were classified into six new groups. The five strains (s1–5) previously described as being responsible for an outbreak in 1989 [7] in the ICU of this hospital were divided among four of the six groups but all four were closely related to each other. Strain 15, isolated in an orthopedic ward 8 months after the ICU outbreak, was found to be identical to strain s4. In contrast, although they were isolated in the ICU 2 months before and 8 months after the outbreak, respectively, strains 12 and 13 were placed in two different groups showing great distances between them and the other groups (Figure 1).

Two strains (16 and 30) isolated in hospital C, located in the Paris area, were placed in two different and new groups, but strain 16 was found to be identical to strain 89, which was the single strain isolated in hospital G, located in Pas-de-Calais.

All but one (34) of the strains isolated within a short period in the rehabilitation institute in Pas-de-Calais were placed in a single and new group. Nevertheless, the one strain isolated in hospital F located in the Paris area was placed in this group. Strain 34 constituted a new group, and the strains isolated in hospitals D and E also constituted two different new groups.

RAPD fingerprints of *E. coli* derivatives and ESβla-producing *P. mirabilis*

E. coli J53-2, *E. coli* MC1601 and *E. coli* derivatives representative of the nine different transferable resistance patterns were submitted to RAPD fingerprinting. As shown in Figure 2, no difference could be observed with use of the primer HLWL74 between the RAPD fingerprints of both plasmid-free *E. coli* strains and the different *E. coli* derivatives. With use of the two other primers, AP4 and MHN1, identical results were obtained (data not shown).

In contrast, the 40 ESβla-producing *P. mirabilis* isolates were classified into six groups based on RAPD fingerprinting with the primer HLWL74, into eight with the primer AP4 and into 10 with the primer MHN1 (Figure 3). After combining the fingerprints produced by each of the three primers for each strain, the 40 *P. mirabilis* strains were classified into 12 fingerprinting profiles (Figure 1). Profile a was observed in

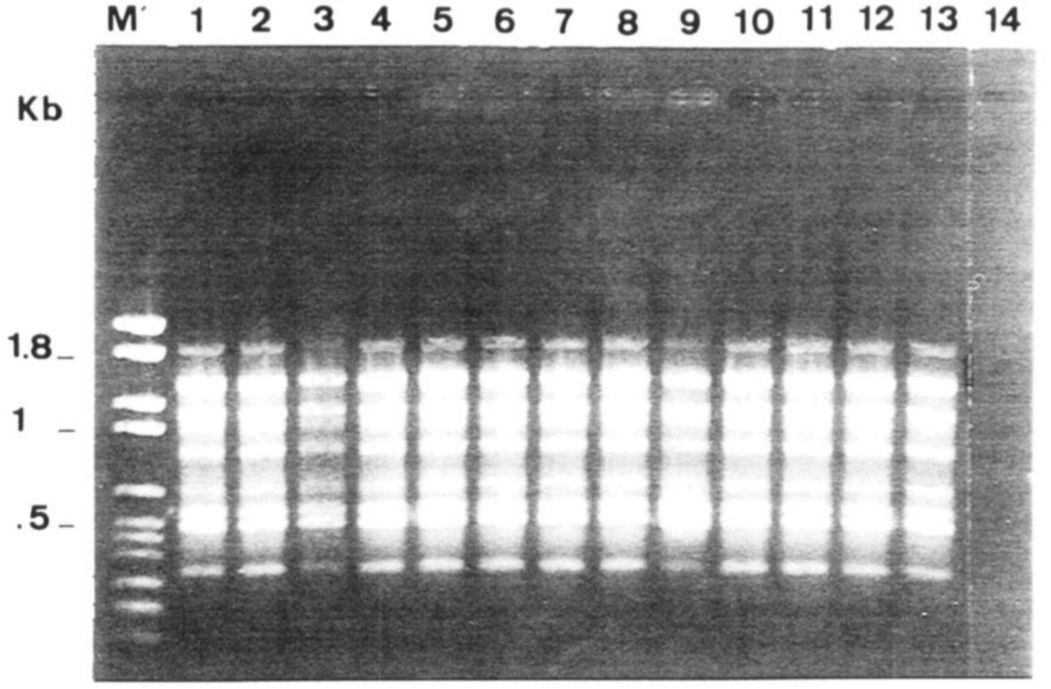

Figure 2 RAPD fingerprints obtained with the primer HLWL74 of plasmid-free *E. coli* and isogenic *E. coli* derivatives harboring the plasmids encoding the nine representative transferable resistance patterns. Lane 1: *E. coli* J53-2. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11: *E. coli* derived from *P. mirabilis* 18, 19, s3, 15, 16, 1, 7, 13, 17 and 30 respectively. Lane 12: *E. coli* MC-1061. Lane 13: *E. coli* derived from *P. mirabilis* 14. Lane 14: negative control. M′, molecular weight marker. Molecular sizes are indicated in kb on the left.

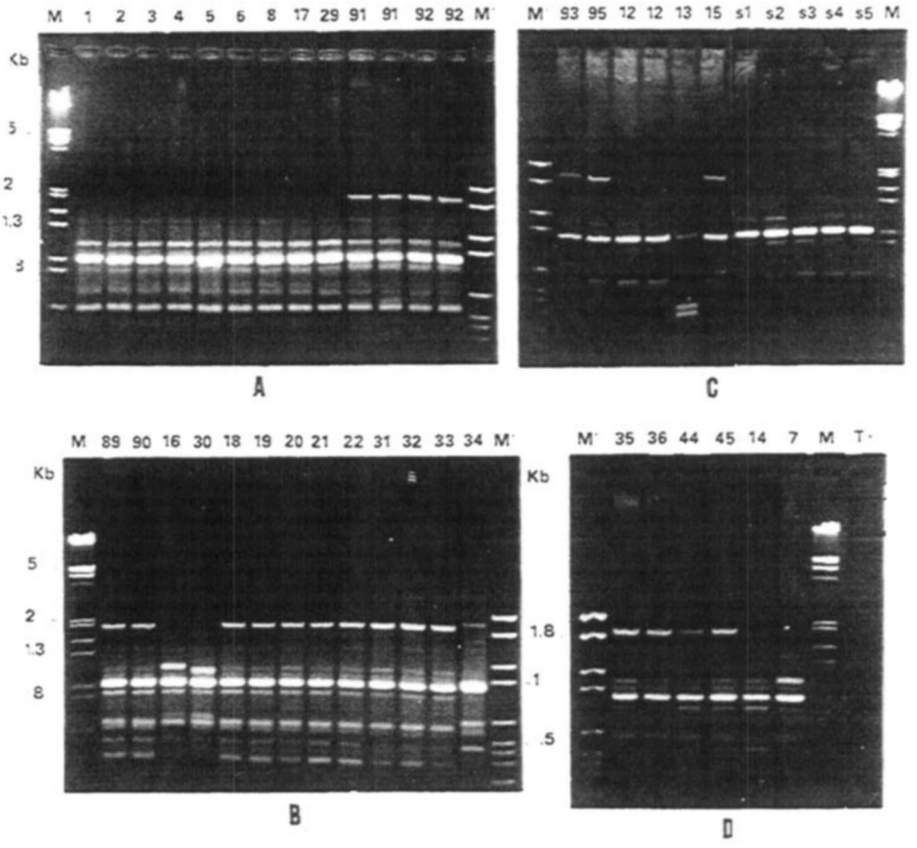

Figure 3 RAPD fingerprints of 40 ESβla-producing *P. mirabilis* strains obtained with the primer MHN1. The strain number is indicated above. M and M′, molecular weight markers. Molecular sizes are indicated in kb on the left. T−, negative control. The same fingerprint was obtained with *P. mirabilis* 1, 2, 3, 4, 5, 6, 7, 8, 17, 29, s1, s2, s3, s4 and s5. Another fingerprint was shared by *P. mirabilis* 91, 92, 95 and 15, and another by *P. mirabilis* 89, 90, 18, 19, 20, 21, 22, 31, 32, 33, 35, 36, 44 and 45. Strains 16, 30, 93, 15, 14, 34 and 13 each displayed a specific fingerprint.

eight (1, 2, 3, 4, 5, 6, 8 and 29) of the 13 strains isolated in hospital A, in strain 17 isolated in hospital E, and in the five strains (s1–5) responsible for an outbreak in hospital B. Profile b was found in all the strains isolated in the rehabilitation institute in Pas-de-Calais, in strain 90 isolated in hospital F, and in two strains (91 and 92) isolated in hospital A. Profile c was displayed by only one strain (15 in hospital B), while profile d was displayed by two strains, one (89) isolated in hospital G in Pas-de-Calais, and one (95) of the strains isolated in hospital A. The remaining profiles, e, f, g, h, i, j, k and l, were each observed in single strains: 93, 12, 13, 30, 34, 16 and 7, respectively.

DISCUSSION

Clinical involvement of ESβla-producing *P. mirabilis* has remained rare, in comparison with ESβla-producing *K. pneumoniae* [21,22]. This study shows that only one strain was isolated in four hospitals and two strains in two hospitals during the 7-year survey period. Nevertheless, the clinical impact of such strains can be important locally, since 13 strains were found in hospital A and 13 others in the rehabilitation institute H. In the first case, these strains were isolated within a period of 4 years, whereas those in the second case occurred within 3 months, strongly suggesting the spread of an ESβla-producing *P. mirabilis* strain in this institute. Despite an outbreak in 1989 including five strains previously demonstrated as epidemiologically and phenotypically related in hospital B [7], only sporadic isolations of other ESβla-producing *P. mirabilis* strains were observed afterwards in this hospital.

According to inhibition zone diameters of cefotaxime, ceftazidime and aztreonam, the studied ESβla-producing *P. mirabilis* strains showed more commonly a cefotaximase than a ceftazidimase phenotype, as was the

case with the first ESβla identified in *K. pneumoniae* [1,3,17]. As observed in other enterobacterial species [2,4,5], the transferable plasmids encoding ESβla in *P. mirabilis* also encode multiple antibiotic resistance. Nevertheless, despite the use of selecting antibiotics corresponding to classical transferable antibiotic resistance markers, no *E. coli* derivatives were obtained from three ESβla-producing *P. mirabilis* isolates. This failure could be related to the low frequency of *P. mirabilis* plasmid transfer which has already been described for this species [7]. From another ESβla-producing *P. mirabilis* strain, no transconjugant could be selected on medium containing ampicillin or cefotaxime, whereas such could be selected on medium containing kanamycin. Moreover, these transconjugants did not exhibit a β-lactam susceptibility profile consistent with the production of ESβla. This phenomenon might be due to the presence of a second non-motile and ESβla-encoding plasmid or to the localization of the ESβla-encoding gene on the chromosome, as was reported for TEM-12 in an *E. coli* strain [23].

To assess the clonal relatedness of the 40 ESβla-producing *P. mirabilis* isolates, we chose two typing systems so that the markers studied were totally different. For the quantitative antibiogram typing, the marker consisted of the expression of chromosomal- or plasmid-mediated genes encoding antibiotic resistance. For the RAPD fingerprinting, the marker concerned the random amplification of chromosomal DNA fragments. We demonstrated that the DNA of the plasmids carried by ESβla-producing *P. mirabilis* isolates was not a template for the primers used, since an identical RAPD fingerprint was found for plasmid-free *E. coli* and *E. coli* derivatives harboring the transferable plasmids. Despite the difference of the studied markers, the strain classifications obtained by the two typing systems appeared generally concordant. Indeed, the majority of the strains considered as identical by the euclidean distance method displayed the same RAPD fingerprint, and the strains showing the greatest distance between them displayed specific RAPD fingerprints. However, even with the use of three random primers, which increases the discriminatory power of RAPD fingerprinting, this method appears less discriminatory than the quantitative antibiogram typing system (12 fingerprints versus 19 groups). With the five ESβla-producing *P. mirabilis* isolates obtained over a period of 1 month in an ICU of hospital B, which were considered to result from the spread of a unique strain, according to the clinical epidemiologic data, the resistance phenotype, the plasmid content and the kinetic parameters of the ESβla produced [7], we found, in this study, that these strains were identical by the RAPD typing system but classified into four different, but closely related, groups by the quantitative antibiogram typing system. This observation suggests that a typing system based on the bacterial expression, such as antibiotic resistance, could be too sensitive in comparison with a typing system based on bacterial structure, such as chromosomal DNA. Indeed, quantitative antibiogram typing identifies differences in isolates which belong to a same clone but are involved in an epidemic occurring over a relatively long period. On the other hand, the quantitative antibiogram typing system can be confusing, since it placed strains (s4 and 15 or 89 and 16) in the same group, while the RAPD typing system distinguished them. Given the localization on plasmids of the majority of antibiotic resistance-encoding genes, two strains can be defined as identical by quantitative antibiogram typing because they carry the same plasmid, while being different in the system of random chromosomal DNA amplification. This study also showed that strains which were placed into different and distant groups (91, 92 and all the strains isolated in the rehabilitation institute or 95 and 89, for example) could have the same RAPD fingerprint. This phenomenon might be consistent with the presence of different plasmids in the same strain.

These observations emphasize the usefulness of using two different typing systems to analyze the clonal relatedness of strains. Applying this approach in this study, we showed that all but one of the strains isolated in the rehabilitation institute in Pas-de-Calais were identical. The single strain isolated in hospital G, also located in Pas-de-Calais, was different from the strains isolated in the rehabilitation institute. In contrast, the single strain isolated in 1993 in hospital F located in the Paris area was found by both typing systems to be identical to the epidemic strain in the rehabilitation institute. This finding is interesting but not surprising, because patient transfer from rehabilitation institutes to hospitals is common. Nevertheless, this strain was not responsible for any epidemic spread in hospital F.

In hospitals A and B, a similar situation was observed, namely the presence of an epidemic strain, and the presence of sporadic strains. Interestingly, both epidemic strains displayed the same RAPD fingerprint and this could suggest an association with epidemic potential. The single strains isolated in the other hospitals appear unique by both typing systems. This was notably the case with the first strain (14) of ESβla-producing *P. mirabilis* isolated in France.

In conclusion, when a number of ESβla-producing *P. mirabilis* isolates were obtained in a single hospital during the 7-year survey, these isolations represented essentially the spread of a same strain, whereas when a single or few isolates were obtained, these isolates each represented a unique strain. Thus, the transfer of

ESβla-producing *P. mirabilis* strains between hospitals does not appear to be common.

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